Alleviation of Metabolic Endotoxemia in Adults with Metabolic Syndrome with Milk Fat Globule Membrane
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I. Objectives

Our preclinical evidence shows that phospholipid-rich milk fat globule membrane (MFGM) attenuates lipopolysaccharide-induced increases in gut permeability and pro-inflammatory cytokines. MFGM also attenuates inflammation in association with a prebiotic and/or antimicrobial activity that modulates microbiota composition. Our ***central hypothesis*** is that MFGM-enriched dairy milk compared with a matched milk beverage containing coconut and palm oil (control) decreases metabolic endotoxemia and improves glucose tolerance in metabolic syndrome (MetS) adults by increasing gut barrier integrity in association with alleviating gut dysbiosis and inflammation. To test this, we will fulfill the following ***objectives***: ***1) MFGM-mediated improvements in gut barrier function.*** We *hypothesize* that MFGM-enriched milk decreases MetS-associated endotoxemia by alleviating gut dysbiosis and improving barrier integrity, and 2) ***Improvements in glucose tolerance by MFGM.*** Our *hypothesis* is that MFGM-enriched milk improves glycemic control in association with attenuating TLR4/NFκB-dependent inflammatory responses.

II. Background and Rationale

Endotoxemia is prognostic of metabolic health. Gut-derived bacterial endotoxins (i.e. LPS derived from the cell membrane of Gram-negative bacteria) trigger endotoxemia and induce NFκB inflammation following their ligation of TLR4. Key determinants of metabolic endotoxemia are intestinal bacteria overgrowth (i.e. gut dysbiosis) and diminished gut barrier integrity. Consequently, gut-derived endotoxin translocation triggers TLR4/NFκB-dependent inflammatory responses (e.g. TNFα, IL-1, IL-6) to provoke metabolic derangements (e.g. insulin resistance, lipogenesis).[1] Preventing gut-derived endotoxin translocation is therefore critical for alleviating endotoxemia. Although the mechanism by which dairy-rich diets protect against MetS is under investigation, our preclinical studies support gut-level antiinflammatory benefits of MFGM,[2] a protein-lipid complex that surrounds the fat globules in milk. Thus, we propose a translational study in MetS persons using dairy milk formulated to be enriched with MFGM to alleviate endotoxemia. We expect that MFGM will suppress gut barrier dysfunction and endotoxin-inducing inflammatory responses in the host attributed to LPS-TLR4-NFκB activation.

Dairy milk provides gut-level cardiometabolic benefit. Controlled studies aim to establish the cardiometabolic mechanisms of dairy foods that have been suggested by observational studies.[3] Chronic feeding studies generally support this concept, but many are difficult to interpret because of the complex composition of dairy milk. Likewise, displacement of "pro-disease" non-dairy foodstuffs by dairy foods also limits interpretation. The Bruno Lab has circumvented these challenges, in part, by studying the controlled administration of dairy milk or its constituents on postprandial metabolic health.[3-6] For example, our recently completed randomized controlled trial in prediabetic adults attributes a portion of the cardiometabolic benefits of dairy directly to its casein and whey protein fractions (under review).[7,8] Specifically, dairy proteins lower cardiovascular risk, likely by a mechanism involving the gut-vessel axis that increases cholecystokinin to limit hyperglycemia-mediated oxidative stress that otherwise impairs nitric oxide-dependent vascular endothelial function. However, separate gut-level benefits of dairy milk are likely attributed to dairy fat-derived MFGM.[2] This is consistent with our studies in which MFGM exhibited antiinflammatory benefit in rodent models.[2,9] Thus, through an innovative controlled feeding study with a matched placebo, we will have

a novel opportunity to translate the benefits of MFGM to improve gut barrier function and attenuate pro-inflammatory responses at the gut and systemically.

MFGM is a unique milk fat-derived antiinflammatory constituent. The MFGM is the membrane (trilayer) surrounding secreted milk fat droplets.[10] It originates from the apical surface of mammary epithelial cells and is comprised of a unique triple phospholipid and cholesterol layer embedded with proteins and glycoproteins. The genes that synthesize MFGM are conserved across species and its composition differs little between-species. Notably, phospholipids account for 1% of total milk lipid, with ~one-third each from sphingomyelins, phosphatidylcholines, and phosphatidylethanolamines. Although MFGM is present in most dairy foods, churn buttermilk is quite rich in this membrane fraction. Indeed, churning of milk fat disrupts the membrane surrounding the fat globules. This results in the coalescence of free fat that ultimately yields butter. In contrast, buttermilk is the aqueous phase (i.e. MFGM-containing portion). MFGM constituents (e.g. polar lipids, gangliosides, proteins) improve intestinal health[9] and exert antiinflammatory activity systemically and at the gut.[11-14]

MFGM protects against endotoxin-induced gut permeability. MetS is characterized by increased "gut leakiness" consistent with our preclinical studies in obese mice showing that endotoxemia occurs in association with decreased expression of intestinal tight junction proteins (e.g. occludin, zonula occluden, claudin).[15,16] In contrast, our dietary interventions with a functional food (green tea) fully protected against the loss of gut barrier integrity and normalized serum endotoxin levels to attenuate hepatic TLR4/NFκB inflammation and oxidative stress.[15,16] Similarly, the polar lipids present in MFGM provide resistance to epithelia stress.[12,17-19] These bioactivities are important, because they are expected to support MFGM to alleviate metabolic endotoxemia in the proposed clinical nutrition intervention, consistent with a mechanism of improving gut barrier integrity (*Obj 1*) and attenuating TLR4/NFκB inflammation and glucose intolerance (*Obj 2*).

Gut dysbiosis mediates endotoxemia in MetS. Inflamed intestine has high oxidant stress,[20] which contributes to endotoxemia by permitting increased LPS absorption and transcytosis of enteric bacteria. Oxygen gradients also decrease steeply from the upper to lower gastrointestinal tract and increase from the lumen to the more vascularized epithelial layer. Oxygen regulates microbial colonization with more oxygen-tolerant bacteria in the mucosa (e.g. *Helicobacter*) whereas obligate anaerobes (e.g. *Firmicutes*) are luminal.[21,22] Oxygen gradients can also contribute to microbial biogeography by influencing metabolite production and modulating redox effectors (e.g. nitric oxide, hydrogen sulfide, reactive oxygen species) of either bacteria or host origin.[23] Importantly, reactive oxygen species generated by the intestinal epithelium[24] function to exert redox-responsive pro-inflammatory cell signaling and disrupt gut barrier integrity.[25,26]

Our studies in obese mice demonstrate the importance of the microbiome to manage endotoxemia in relation to MetS.[27] We show that endotoxemia is mediated by gut dysbiosis in association with increased intestinal inflammation that impairs gut integrity. In our first approach, we showed that obesity-associated endotoxemia is attributed to increased "gut leakiness" (based on FITC-dextran absorption) that causes higher levels of endotoxin in the portal vein circulation. We next showed that gut dysbiosis is associated with an obese phenotype. Specifically, obese mice had an increased ratio of *Firmicutes:Bacteroidetes* and decreased microbial diversity. This occurred in association with decreased predicted functions relating to the synthesis of short chain fatty acids (SCFAs; i.e. butyrate, propionate. These are important microbial metabolites that promote the expression of intestinal tight junction proteins[28] by increasing the stability and transcriptional activity of hypoxia-inducible factor-1α (HIF1α).[29] Likewise, microbial functions relating to LPS biosynthesis were increased in obese mice. We also report that intestinal TNFα, iNOS, MCP-1, and TLR4 mRNA levels and other genes comprising this receptor (CD14, MD2) were all increased in obese mice compared with lean mice (data not shown). Together, especially in the context of the benefits demonstrated by our function food (green tea), targeting gut health is critical to ameliorating endotoxemia by improving microbiota composition and microbial functions that drive endotoxin synthesis and translocation.

MFGM can play a key role to improve microbiota composition and barrier function. Studies in rat pups show that MFGM supplementation improved gut development (e.g. intestinal growth, numbers of Paneth and Goblet cells, and expression of tight junction proteins) compared to control formula.[30] Importantly, the observed benefits of MFGM were similar to those among rat pups reared with mother's milk. Likewise, both MFGM and mother's milk similarly shifted gut microbiota populations compared with formula-fed controls, and they protected against *Clostridium* difficile toxin-induced intestinal inflammation. Microbiota analysis revealed that MFGM and mother's milk pups had greater species richness and evenness compared with controls. In addition, MFGM supplemented pups had greater abundance of *Lactobacilli* compared with controls, but not to the extent observed in pups fed mother's milk. This supports the potential of MFGM to enhance gut barrier integrity by increasing SCFA generation. In piglets, during the postnatal period, dietary supplementation of milk fat and MFGM was shown to modify intestinal protein digestion, modulate immune system maturation, and significantly alter gut microbial populations (e.g. increases in *Proteobacteria* and *Bacteroidetes*; decreases in *Firmicutes*).[31] The latter supports MFGM-mediated improvements in microbiota composition to favorably improve gut physiology.

In addition, based on these gut-level benefits that help to resolve gut dysbiosis by increasing proportions of "healthy" commensal bacteria, several important outcomes are also expected. For example, increased generation of microbial-derived SCFAs would not only ameliorate "gut leakiness" by upregulating tight junction proteins,[28] but increased butyrate also improves barrier function by increasing the secretion of glucagon-like peptide-1 (GLP-1). This incretin helps to alleviate glucose intolerance by potentiating insulin sensitivity.[32-34] Because pancreatic β-cell function, and hence insulin secretion, deteriorates beginning with prediabetes, MFGM would be expected to increase GLP-1 secretion, thereby alleviating dysregulated glycemic responses that provoke MetS risk.

Summary. This overview demonstrates that: 1) low-grade inflammation in MetS is attributed to endotoxemia in association with poor gut barrier integrity, 2) endotoxemia is causally-related to the initiation of obesity and insulin resistance[35] and circulating endotoxin is prognostic of progressive liver function deterioration,[36] 3) MFGM constituents provide antiinflammatory and prebiotic activity at the gut and attenuate endotoxemia, and 4) increased SCFAs from commensal bacteria of the gut not only improve gut integrity, but also promote intestinal incretin secretion to help manage glucose intolerance. Thus, our translational studies in MetS persons are expected to provide novel evidence that MFGM exhibits antiinflammatory activity to enhance glucose tolerance by attenuating endotoxemia. These findings would be of *significance* to public health because could they help to establish that dietary fat "quantity" is not necessarily detrimental to MetS risk, but rather fat "quality" potentiates host health due to enhanced gut barrier function. This would lead to evidence-based recommendations to help reverse course of dietary trends indicating that dairy consumption decreases in an age-dependent manner and that ~90% of Americans fail to meet the daily recommended intake of dairy.[3]

Anticipated Results

We expect the intervention to result in high compliance (>95%), consistent with our rigorous approach to provide all foods and test beverages.

In *Obj 1*, MFGM is *expected* to time-dependently decrease serum endotoxin (i.e. d 0 to d 14), and lower day 14 endotoxin compared with coconut and palm oil-enriched milk. MFGM will also attenuate meal challenge-induced increases in gut-derived endotoxemia. These benefits will correlate with improved gut barrier function as indicated by: 1) decreased urinary recovery of sugar probes (i.e. increased gut integrity), 2) increased fecal SCFA levels; these potentiate intestinal junction protein expression,[28] and 3) an alleviation of gut dysbiosis (e.g. microbial diversity, *Firmicutes:Bacteroidetes* ratio). Indeed, we expect reduced gut bacteria abundance. This is important because intestinal bacteria overgrowth provokes metabolic disease.[37,38] Likewise, MFGM-mediated

prebiotic effects are anticipated to increase commensal bacteria (e.g. butyrate producers) and its anti-microbial activities are expected to reduce pathogenic populations (e.g. pyrogenic bacteria, decreased LPS synthesis).

In *Obj 2*, we *expect* that MFGM will improve glucose tolerance based on decreased fasting glucose (d 14) and attenuated postprandial glycemic responses following the test meal challenge. This will be attributed to antiinflammatory activities of MFGM at the gut (i.e. decreased calprotectin and myeloperoxidase) and on host expression of TLR4/NFκB genes (e.g. TLR4, IL-6). These antiinflammatory benefits are important because they potentiate intestinal GLP-1 and GIP secretion. These incretins improve insulin sensitivity and alleviate glucose intolerance by enhancing pancreatic β-cell function, and hence insulin secretion.[32-34] Decreased TLR4/NFκB signaling will also help to limit metabolic derangements that mediate glucose intolerance in MetS.[1]

Pitfalls and Alternatives

Recruitment. We acknowledge the challenge to recruit MetS adults for a nutrition study. Consistent with our studies,[39-41] we will consult the recruitment office of the OSU CCTS for assistance. They maintain a participant database (>126K registrants) and provide marketing support for study recruitment (e.g. social media, printed flyers, e-newsletters reaching ~100K faculty/staff and students at OSU). If difficulty is encountered to recruit MetS persons, we can modify our approach to focus on persons with prediabetes. *Endotoxemia and Gut Integrity.* We plan to consider endotoxin levels as a covariate in relation to other study endpoints. Also, while we are experienced in measuring endotoxin,[15,16,39,42,43] we are mindful of the experiences of others where these measurements were problematic.[44] If necessary, we will use an alternative assay for LPS as we described,[39] and/or measure surrogate markers of endotoxin exposure (LPS binding protein, soluble CD14) as we described.[42,43] There is the possibility that MFGM may reduce circulating endotoxin without affecting gut integrity. This would indicate that MFGM regulates endotoxemia likely by reducing populations of Gram-negative bacteria or functions of bacteria relating to LPS biosynthesis (to be tested in *Obj 1*) or alleviating transcellular absorption of excess LPS within chylomicrons. If MFGM does not alter *taxonomic composition of the microbiota*, metagenomic and metatranscriptomic analysis[45,46] could be performed to define microbiota functionality. If MFGM does not improve *glucose tolerance*, we will assess triglyceridemia consistent with milk sphingolipid attenuating hepatic triglyceride in response to a high-fat diet.[47] In the event that gut inflammation is unaffected, we will consider untargeted metabolomics of archived fecal samples to identify metabolite patterns in response to MFGM. Lastly, our approach to evaluate postprandial responses considers "net" AUC to reflect metabolite concentrations that increase and/or decrease relative to baseline. If this approach lacks sufficient sensitivity to detect between-treatment differences, we will calculate incremental AUC scores to only consider those changes to rise above baseline values.

Significance of the Research and Potential Benefits

Our "whole food" approach that matches nutrient profiles between study arms will maximize translation of the benefits of full-fat milk that are driven, at least in part, by MFGM. The dose of MFGM at 10-times that in whole milk also reflects that consumers obtain MFGM from dairy foods other than milk. As major outcomes, we expect to demonstrate novel evidence that MFGM-enriched dairy milk decreases metabolic endotoxemia and improves glucose tolerance in MetS adults by increasing gut barrier integrity in association with alleviating gut dysbiosis and inflammation. This will be of *significance* because MetS affects ~35% of Americans,[48] and is driven by inflammation attributed to poor gut health that leads to excess absorption of bacterial endotoxins. Thus, dietary management of endotoxemia with dairy milk could significantly impact the incidence of MetS while helping to explain outcomes of observational studies suggesting benefits of full-fat milk for weight management.[49] Ultimately, this study could help to debunk long-standing dogma that targets full-fat

dairy for energy reduction[50] and support evidenced-based recommendations as part of forthcoming *Dietary Guidelines for Americans*.

III. Procedures

A. Study Design

We will enroll male and female MetS adults (n = 24; 18-65 y) to complete a *2-arm, double-blind, randomized controlled, crossover trial*. They will be randomized in 4-unit blocks to receive, for 14 d, a controlled diet with dairy milk (3.5% fat; 3 servings/d) enriched with MFGM-derived phospholipid or a matched dairy milk that instead contains coconut and palm oil (control). We will provide *all* foods during each study period to ensure weight maintenance and to increase homogeneity of gut and host responses (see *Dietary Control*).

Anthropometrics and blood pressure will be assessed at d 0, 7, and 14. Prior to (d 0) and after each 2-wk arm (d 14), a fasting blood sample will be collected to assess serum endotoxin and metabolic chemistries (glucose, insulin), and TLR4/NFκB-dependent genes from isolated peripheral blood mononuclear cells (PMBCs). A breath sample will be collected to assess the correlation analysis of plasma metabolic biomarkers. After the 2-wk intervention, from fecal samples collected on d 13, we will assess microbiota composition and function, SCFAs, and intestinal inflammatory markers (calprotectin, myeloperoxidase). During this period, participants will also record daily stool characteristics using a 7-point Bristol Stool scale. On d 14, participants in the fasted state will receive a high-fat/high-glucose meal challenge to induce gut-derived endotoxin translocation (described below). At 30-min intervals for 3-h, we will evaluate circulating endotoxin, glucose, and insulin; TLR4/NFκB-dependent genes will be assessed from PMBCs at 0 h and 3 h. Gut permeability probes will be co-administered with the test meal challenge, and 24-h urine will be collected to assess gut barrier integrity. Participants will then undergo a 2-wk washout prior to receiving the alternative treatment and completing all procedures in an identical manner.

B. Study Subjects

Enrollment Criteria. Participants will be recruited from Columbus, OH area with assistance from the OSU CCTS (see *Facilities*) consistent with our studies in MetS persons.[41] Male and female adults with MetS (18-65 y) having no history of liver or cardiovascular disease or cancer will be enrolled. They will have ≥3 established criteria for MetS,[51] with these specific parameters to improve study homogeneity: *i)* glucose (>100 mg/dL), *ii)* waist circumference (>89 or 102 cm for F/M), and *iii)* HDL-C (<50 or <40 mg/dL in F/M). Major *exclusion criteria* include: Blood pressure >140/90 mmHg; unstable body mass (±2 kg over prior 3-mo) vegetarian; food allergies or lactose intolerance; user of dietary supplements or probiotics (within past 1-mo); pregnancy, lactation, changes in birth control (within 6-mo); any gastrointestinal disorders; chronic diarrhea; smoker; excess alcohol (>2 drinks/d); excess aerobic exercise (>7 h/wk); recent antibiotic or anti-inflammatory agent use;.

Recruitment. We will recruit participants through posted flyers, e-mail, electronic and newsprint advertisements (e.g. campus student and faculty/staff newspapers, local and regional newspapers), word of mouth, and through use of ResearchMatch (OSU CCTS). The posted advertisements will instruct interested participants to call the study center (Bruno Laboratory, Department of Human Sciences) or complete an online pre-screening survey to obtain further information. During the phone-in hours, a trained individual (i.e. project coordinator or graduate assistant) will be available to describe the study and determine preliminary qualification by conducting a scripted phone interview (e.g. do you take dietary supplements?, do you smoke?; see *Phone Script* attachment). The individual will record answers and assess whether the person calling is likely or not to be an

acceptable study participant. If the caller and the interviewer agree that the caller should participate, the prospective participant will be invited to a screening meeting, where the study will be fully described, and the individual will be provided a consent form to complete prior to any involvement in the study procedures. Data collected during this phase will include participant's age, health status, physical activity, and contact information (see *Informed Consent* attachment). Recruiting efforts through ResearchMatch will utilize a strategy whereby registered individuals in the ResearchMatch database can be searched for against their non-identifiable volunteer profile in the system. Unidentified individuals meeting search criteria will then be forwarded an electronic recruitment message (see *ResearchMatch Recruitment Message* attachment) that identifies them as a potential match for study participation. The secure ResearchMatch clearinghouse will route this standard notification that provides specific study content (i.e. content similar to that of a posted advertisement) to each of these potential ResearchMatch volunteers who will then have the option of replying "yes", "no", or not respond through a set of quick links available in this notification. ***Note:*** This message will not include the study's direct contact information (e.g. email, phone) as ResearchMatch will measure the response rate through the clearinghouse's quick links made available in this electronic message. The response rate metrics will be made available to researchers through their ResearchMatch dashboard as well as the Institutional Liaison dashboards. By responding "yes", the volunteer has authorized ResearchMatch to release their contact information to the researcher responsible for the study. This information will be made available on the researcher's ResearchMatch study dashboard. The researcher will be responsible for managing this contact information as specified in the IRB-approved study protocol. ResearchMatch will also be collecting aggregate data regarding the status of ResearchMatch volunteers within the study. ResearchMatch volunteers consent to this within the ResearchMatch volunteer agreement. The ResearchMatch enrollment continuum will allow researchers to indicate where the volunteer currently stands within the recruitment process and thus helps researchers monitor the utility and effectiveness of this recruiting tool. Research access to recruit through ResearchMatch will last only as long as the duration of IRB-study approval with the expiration date of ResearchMatch being identical to the end-date of OSU IRB approval. Researchers will be able to submit current IRB-approval letters for the lifetime of the study and thus provide evidence of successful continuing review applications. If an unintentional lapse in time occurs and the research is not able to submit this continuing review evidence via ResearchMatch, stored ResearchMatch data will not be deleted, but the researcher will not have access to this information until a current IRB-approval letter is uploaded and routed to the Institutional Liaison for review. A complete description of ResearchMatch along with the most current IRB approval from Vanderbilt University (i.e. this is the site where ResearchMatch was developed and its secure computer servers are housed) has been attached.

Power Calculation and Data Analysis. Dr. Brock (biostatistician; see *Letter of Support*) performed a power analysis using endotoxin (d 14) as the primary outcome. No human studies have examined MFGM on endotoxemia. Thus, we used our data from MetS and healthy persons in which fasting endotoxin was measured 5-times at 2-wk intervals. On average, endotoxin was >2-times higher in MetS ($P<0.0001$), and did not differ ($P>0.4$) by time or gender. We then calculated the mean difference in endotoxin between healthy and MetS subjects (16.4 ± 7.8 vs 32.4 ± 4.4 EU/mL, CV =48% vs 15%). Power analysis indicated that only 8 subjects/group would be required to obtain 95% power ($\alpha = 0.05$) using the larger of the two CVs. For the proposed study in only MetS persons, we estimated that MFGM would result in a conservative improvement of 60% of the difference in endotoxin in the MFGM vs control phases: ((32.4-16.4) x 0.6 = 9.6 EU/mL); CV = 32% (SD = 10.4). Thus, we would have ~90% power ($\alpha = 0.05$) with 14 MetS persons completing both study arms. Our targeted enrollment of 24 MetS persons (12F/12M) is therefore expected to be powered strongly to examine MFGM on endotoxemia. This will also account for attrition (estimated at 10%) and provide sufficient power to consider gender x treatment interactions.

Most data will be analyzed by linear mixed effects models with random effect for subjects (to account for repeated measures) and fixed effects for treatment, gender, time period, and their interactions. Multivariate regression will define correlations between variables with consideration of potential covariates. Significance for all analyses will be set at $P<0.05$.

C. Measurement/Instrumentation

Anthropometric Parameters and Blood Pressure. At screening, day 0, 7, and 14, participants will rest for 15 minutes prior to determining blood pressure using an automated cuff. BMI will be calculated from height determined from a wall-mounted stadiometer and weight from a calibrated scale. Waist circumference will be assessed at the level of the umbilicus using a nonflexible measuring tape.

Blood Chemistries. Fasting plasma glucose and lipids (total cholesterol, HDL-C, triglyceride) will be measured by clinical assay (Pointe Scientific) at screening, day 0, and day 14. Fasting insulin will be measured by ELISA (ALPCO) at day 0 and day 14. TLR4/NFκB-dependent genes from isolated peripheral blood mononuclear cells (PMBCs) will be assessed. For each arm of the study, we will measure circulating glucose and insulin at 0, 30, 60, 90, 120, 150, and 180 min following the ingestion of the glucose beverage. TLR4/NFκB-dependent genes will be assessed from PMBCs at 0 h and 3 h.

Breath Analysis. Breath samples will be collected using a sterile, single use mouth piece into a sterile breath collection bag. Participants will be instructed not to brush their teeth with toothpaste, gargle with mouthwash, chew gum or eat any mints the morning of each test. Breath samples will be analyzed within one hour using a mass spectrometry-based breath analysis technique.

Preparation of Milk Beverages.

Isolation of MFGM Phospholipids. The main component for our formulation, the MFGM containing ingredient (i.e. buttermilk powder), will be prepared as described.[52] Sweet cream will be prepared from milk obtained from the OSU Waterman Dairy Farm using a cream separator after pasteurization. After tempering (16 h, 4°C), cream is churned using a continuous pilot scale butter churn, and butter fines are then removed by filtration. A pilot plant-scale system using two spiral polymeric membranes fitted in parallel on the module (10 kDa, 11.33 m^2 total surface area) will be used to concentrate buttermilk. This is performed at 15°C with transmembrane pressure of 500 kPa. Ultrafiltration is performed until a 10-fold volumetric concentration factor is achieved. Diafiltration (DF) is then performed by adding water continuously at 15°C to the feed tank to replace the removed permeate until a 5-fold DF factor is achieved. The final retentate is then spray-dried to obtain buttermilk powder having ~20-40 times the phospholipid content found in commercial milk.

MFGM and Coconut/Palm Oil Beverages. Our approach entails formulating MFGM- and coconut/palm oil-enriched milks with 10-times the normal concentration of total phospholipids. MFGM (isolated above as buttermilk powder) contains ~2 g of phospholipid per 10 g of solid. Based on commercial whole milk containing 0.2-0.3 g/L phospholipid, the addition of 5-10 g MFGM (i.e. buttermilk powder) would yield a final concentration of 2-3 g/L of total phospholipid in our formulated beverage. Thus, milk beverage manufacture, based on our in-house pilot tests, would be as follows:

MFGM Milk. A modified cream with milk phospholipids will be prepared using 40% butter fat, 10% MFGM ingredient (i.e. buttermilk powder), and 50% skim milk. Ingredients will be homogenized and added to skim milk to prepare milk containing 3.5% total fat (*w/w*).

Coconut and Palm Oil Milk. A modified cream will be prepared by homogenizing 12.5% coconut oil, 37.5% palm oil, and 50% skim milk. This will yield a milk beverage matched to MFGM milk for

saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, but not enriched with phospholipids.

Both beverages will be homogenized (2-stage at 1500 and 500 psi), pasteurized, bottled in single-use servings under aseptic conditions, and refrigerated until use. We anticipate preparing up to 250 gallons of milk beverages to complete the study. Beverages are shelf-stable for 21 d and will be prepared in sufficient volume on a weekly basis proportional to the number of participants enrolled. Phospholipid content will be verified in the Jiménez-Flores Lab to be ±5% of expected concentrations with each prepared batch. Under the aforementioned manufacturing conditions, the energy and nutrient content of both beverages will be matched (3.2% protein and 3.5% fat, *w/w*). However, the MFGM beverage will contain 3 g/L MFGM phospholipid. The coconut/palm oil-based beverage will contain negligible levels of MFGM phospholipids (0.005% w/w), reflecting that skim milk is not fully devoid of these polar lipids.

Dietary Control. The intervention will be performed in the Human Nutrition Metabolic Kitchen under the auspice of a registered dietitian (PI Bruno). In each 2-wk intervention, participants' diet will be rigorously controlled. All foods will be prepared, packaged, and provided every 3-4 d to supply a weight maintenance (i.e. eucaloric) diet. To assess compliance, participants will return MFGM/coconut/palm oil milk bottles for counting and any uneaten food portions for weighed measurement. Milk beverages will also be formulated to contain para-aminobenzoic acid (PABA; 200 mg/milk serving). Spot urine samples will be collected 5 times during each study arm coinciding when participants pick up test foods. Urinary PABA will be measured by spectrophotometry according to procedures provided by Dr. Mario Ferruzzi (see *Letter of Support*). Separate from this, participants will also keep food logs to document any dietary deviation. Diets will be standardized at 50-60% of energy from carbohydrate with low fiber intakes (~15 g/d) similar to Americans' diets[53] to prevent potential masking of the benefits of MFGM, 15-20% from protein, and 25-30% from fat. Importantly, other than test beverages provided as part of the eucaloric diet, diets will be otherwise devoid of significant amounts of dairy foods, fermented products, and probiotics to prevent confounding effects.

Gut Permeability. On d 14, postprandial endotoxemia and hyperglycemia will be induced by a high-fat/high-glucose challenge meal,[54,55] as described, while also assessing gut barrier integrity. Participants will ingest a controlled meal (~1200 kcal) consisting of 3 slices of toast with 50 g margarine along with a glucose tolerance beverage (75 g) that also contains gut permeability probes [lactulose (5 g), mannitol (1 g), sucralose (1 g), and erythritol (1 g)].[56] In-house pilot tests indicate tolerability of the test meal. With this procedure, endotoxin increases by ~50% within 2 h[54] and increased glucose provokes oxidative stress responses including lipid peroxidation.[55,57] We will collect blood from 0-180 min at 30 min intervals and urine for 24 h to assess study outcomes.

Endotoxin. Serum endotoxin will be measured from fasted participants (0 min) and during the postprandial period (30, 60, 90, 120, 150, 180 min) following ingestion of the sugar solution, using a fluorometric kit as we described.[15,58]

Microbiota Composition. Microbiota composition will be assessed from fecal samples collected on d 13 of each study period. Samples will be extracted in the Bruno lab, and genomic sequencing will be performed in the OSU MCIC core lab (see *Facilities*) using routine procedures. In brief, effects due to MFGM on total bacterial abundance will be assessed using qPCR (i.e. to assess intestinal bacteria overgrowth).[59] Microbiota of each sample will be characterized for diversity and community structure by sequencing 16S rRNA genes using MiSeq. Bioinformatic analysis will be performed using Qiime[60] and bacteria identified by comparing each operation taxonomic unit (OTU) to RDP and Silva databases.[61,62] α-Diversity (OTU richness, Shannon-Wiener diversity index, evenness) will be calculated using Qiime and β-diversity using the Bray-Curtis dissimilarity,[63] followed by multivariate analysis (i.e. PCA, PERMANOVA, ANOSIM, PLS-DA) to determine MFGM effects.

The functional metagenome will be predicted using PICRUSt[64] and PanFP[65] based on reference databases and the KEGG Orthology classification scheme to obtain a functional gene abundance matrix.

We will validate preclinical findings showing increased microbiota potential to generate SCFAs in response to MFGM.[30,31,47] These gut health-promoting metabolites (butyrate, acetate, propionate) will be quantified by LC-MS as we described,[57] with modification,[66] from fecal samples collected on d 13. Quantification will be performed relative to ^{13}C-butyrate, -acetate, or -propionate (internal standards), and normalized to fecal dry matter.

D. Detailed Study Procedures

Overview of Study Procedures. Potential participants who call the study center in an anonymous manner for more information as well as those identified through ResearchMatch will be given a brief description about the study and asked a few questions to determine their eligibility (*see Phone Script* attachment). If they meet the eligible criteria, they will be invited to the study center for a screening meeting. During the meeting, the Informed Consent (see *Informed Consent* attachment) will be explained and provided for them to review. The participant will then be given the opportunity to review the Informed Consent form. If he/she chooses to participate in the study, they will then be asked to provide written consent. After receiving informed consent, the participants' height, weight, waist circumference, and blood pressure will be measured. Additionally, a small fasting blood sample will be collected for blood chemistry analysis. A breath sample will also be collected. If they are not fasted at least 10 hours, they will be asked to come back in the fasted state at time of mutual convenience. These blood results in combination with anthropometric parameters will determine the participant's eligibility. Eligible participants who agree to proceed with the study will then complete two 2-week intervention periods in a randomized order, each followed by a 3-h postprandial trial. We estimate that completion of all study procedures will take ~6-8 weeks per participant. Each step of the study procedure will be discussed in detail below:

Screening Meeting. Potential participants who have met the initial criteria of the study (based on the telephone interview) will be invited to the study center at a mutually convenient time. During this time, the participant and a member of the research team will meet in a private, quiet conference room or office. The individual will be provided the informed consent form, and its contents will be described to the potential participant. The participant will then review it, and if they choose to participate in the study, they will be asked to provide written consent. Although the participant will be asked to sign the informed consent, the participant will be told that they will not be asked to participate if their body measurements, blood pressure, or plasma chemistries (see *Enrollment Criteria*) do not meet the study criteria. If the participant has provided consent, we will then measure the participant's height, weight, waist circumference, and blood pressure. Next, if the participant is fasted for at least 10 hours, we will ask if a trained individual can draw a small blood sample (10 mL; 2 tubes) so that we may measure blood chemistries (glucose, HDL-cholesterol, triglyceride). A breath sample will also be collected for the purpose of the correlation analysis of human plasma metabolic biomarkers. All samples will be coded to maintain participant anonymity. If the participant's anthropometrics, blood pressure, or plasma chemistries do not meet the study criteria, they will be told that they do not meet the study criteria.

Potential participants who meet the criteria will be contacted within a few days after their screening meeting to provide them with their blood and body measurement results and inform them of their eligibility to participate in the study. Consistent with our CLIA exemption, blood results will be provided in a categorical manner (i.e. "normal", "marginally high", "high") rather than providing actual blood concentrations of lab values (see *Subjects Results Sheet*). Potential participants having any blood values outside of the "normal range" will be directed/encouraged to follow-up with their

own physician. Those having body measurements, blood pressure, and blood values within acceptable limits (see Inclusion/Exclusion criteria), will be invited to participate in the study. Subjects will be included or excluded based on a best fit of the inclusion and exclusion criteria (an example of best fit would be if a potential subject says he/she exercises 6 h/wk, which is close to our exclusion criteria of 7 h/wk, they might still be included in the study if they meet all other inclusion and exclusion criteria more closely than other potential subjects). Participants will be read one phone script if they qualify and another phone script if they do not qualify (see *Participant Eligibility Phone Script* attachment). Potential participants who qualify for the study will be communicated a message as follows: "Congratulations! You have been selected for participation in our study based on your blood testing results. A study investigator will be telephoning you to invite you to the testing session. Would you like to know your blood chemistry results?" Subjects not selected for study will be told the following message: "You have not been chosen for our study, but thank you for your interest." This message will be followed by an explanation why they were not chosen, such as lab values outside of the range we are looking for: "Your blood testing data is…..we were looking for participants who had levels less than …..". "We can provide you a copy of your results if you would like…..how would you like us to provide them to you?". "You should also consider sharing these results with your physician."

If a participant is telephoned and is unavailable, a message will be left requesting a callback at a convenient time or that at a member of the study team will try calling again at a later time. No confidential or sensitive information will be shared with third parties or left on answering machines.

Intervention timeline. Each participant will complete a 2-arm (MFGM-Enriched Milk; Coconut/Palm Oil-Enriched Milk), randomized, cross-over study. For this study, each participant will visit the study center a total of 12 times (6 times per study arm, including 1) baseline measurements of anthropometrics, serum glucose, insulin, endotoxin, and pick-up of meals for days 1-3, 2) pick-up of meals for days 4-6, 3) pick-up of meals for days 7-10 and measurement of anthropometrics, 4) pick-up of meals for days 11-14 and stool collection kit, 5) 3 h postprandial testing, and 6) return samples from 24-h urine collection. Interventions will be separated by two weeks (for women, interventions will be separated by 2, 6, 10 etc. weeks to ensure testing will be performed at the same time of the menstrual cycle).

Baseline visit. On day 0 of each trial arm, participants will report in the fasted state (10-12 hours) to the study center for assessment of height, weight, waist circumference, blood pressure, serum glucose, serum insulin, and serum endotoxin. They will receive 3-4 days of meals plus MFGM-enriched milk or coconut/palm oil-enriched milk.

Dietary intervention. Participants will be provided all foods for the 14-d intervention period. After the baseline visit, participants will report back to the study center on days ~6 and 10 to pick up their meals for the proceeding 3-4 days. On day 7, anthropometrics (height, weight, and waist circumference) will also be measured to ensure weight maintenance.

Test trials. At the end of the intervention period (day 14), participants will report back to the laboratory in the fasted state (10-12 hours) for assessment of height, weight, waist circumference, blood pressure, plasma glucose, insulin, gastric inhibitory peptide (GIP), glucagon-like peptide-1 (GLP-1), and serum endotoxin. Participants will return a stool sample collected on day 13 of the intervention period at home. Participants will ingest a sugar solution containing 75 g glucose, which we and others have routinely shown to provoke oxidative stress responses including lipid peroxidation. This will be co-ingested with 1 g sucralose, 1 g erythritol, 5 g lactulose, and 1 g mannitol for assessment of gut permeability. Then, at 30 min intervals during the 3 h postprandial period, we will assess blood markers. During each study visit, participants will have access to drinking water

and use of the restroom as needed. Urine will be collected during the 3 h postprandial period in the laboratory, and participants will be instructed on urine collection for when they return home following their laboratory visit. Participants will return to the study center the following day to drop off their 24-h urine collection tubes. To ensure that the order by which each participant completes each trial is randomized, a random sequence generator (http://www.random.org/sequences/) will be used to determine the order by which each participant will undergo the two trials.

Sugar Probe Test. On the morning of testing at day 14, participants will arrive at the study center after abstaining from food and only consuming water for 10-12 hours. A sugar probe test will be conducted as described.[56] Upon arrival, participants will be asked to empty their urinary bladder. Subsequently, they will then be asked to ingest a drink containing 1 g sucralose, 1 g erythritol, 1 g mannitol, and 5 g lactulose dissolved in 75 g glucose (240 mL). Within 2-hours after ingesting the sugar test beverage, participants will be asked to consume an additional 500 mL of water. Additionally, participants will be provided with standardized sucrose, erythritol, mannitol, and lactulose-free meals (e.g. bagel, peanut butter, scrambled eggs) during the 24-h period. All foods consumed during the 24-hour period will be free of artificial sweeteners. Urine will be collected from 0-5 and 6-24 hours in sterile containers. For each urinary collection period, participants will be provided 2-L urine collection bottles and an insulated reusable bag with ice packs to store collected urine until they return their samples the next day.

Sampling Handling. Once at baseline (d 0), once at d 7, and at each time point of the postprandial trial a blood sample (14 mL, 4 mL, and 20 mL x 7 time points = 140 mL, respectively) will be collected into evacuated blood collection tubes. Collecting a total of 158 mL or 0.67 cups once at baseline, once at d 7, and during the postprandial trial is necessary to ensure adequate amount of plasma and serum for each time point to accurately analyze levels of glucose, insulin, endotoxin, gut hormones, and TLR4/NFκB signaling genes. During blood collection, participants may feel an initial pain when inserting the needle, bruising around the insertion area, lightheadedness, or fainting, which are common when donating blood. However, we do not foresee any additional significant risks for collecting this amount of blood over 3 hours, other than the risks stated previously. Breath samples will be collected in a clean breath collection bag supplied with a sterilized disposable mouth piece. Participants need to breathe into the bag at their normal breathing speed. Generally, three to five regular breaths will be sufficient to fill the breath bag, and there is no risk associated with this procedure.

Throughout the span of the study (~8-12 weeks in duration depending on participant/investigator availability) which consists of four weeks total of controlled feeding, a blood collection at day 0, 7 and 14 and then repeated following a ≥2 week washout period, and 1 screening day, we will be collecting a total of ~324 mL or ~1.37 cups) of blood. Urine will be collected in provided containers (VWR) containing 10% thymol to inhibit bacterial growth. We will then have the participants return their urine samples to the study center or coordinate with study personnel to meet at a public and mutual location to return their samples within 24-h after collection. Feces will be collected using a commercial commode specimen collection system (Fisher Scientific). Briefly, the collection kit consists of the necessary materials (e.g. gloves, waste bag) for participants to easily and hygienically collect their stool without contaminating the sample or themselves. Volumes of urine will be recorded and fecal mass and observations will be recorded based on the Bristol Stool Chart.[67]

During each blood collection, plasma will be obtained by centrifugation and then transferred to cryogenic storage tubes. Serum samples will be obtained by allowing the blood to clot, followed by centrifugation and transfer to cryogenic storage tubes. Tubes will be stored at -80° C until analysis can be completed. Analyses will include plasma glucose, insulin, triglyceride, HDL cholesterol, TLR4, CD14, MD2, MyD88, p65, IL-1, IL-6, IL-8, TNF-α, MCP-1, GLP-1, GIP, and serum endotoxin. Urine will be stored at -80ºC until analysis can be completed. Analysis from urine will include sucralose, erythritol, lactulose, and mannitol. Fecal samples will be stored at -80ºC until analysis can be completed. Feces analysis will include microbiota composition, calprotectin, myeloperoxidase (MPO), and fecal short chain fatty acids (butyrate, acetate, propionate). Remaining plasma, serum, urine, and fecal samples not used for these analyses will be archived for 5 years at -80 C in the event we decide to measure additional inflammatory, antioxidant, or microbiota related markers. Appropriate notation has been made in the informed consent to alert participants that we will be archiving specimens and that they have the right to refuse our use of these specimens for future analyses. Lastly, approval from OSU IRB will be sought via a protocol amendment prior to the analysis of any additional biomarkers not specified herein.

Privacy/Confidentiality. For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant and will only be available to research personnel. Any records containing the names of participants will be stored in a locked filing cabinet or on a password protected computer in the PI's laboratory or office. Research personnel under the supervision of the PI and the PI himself will be the only individuals that have access to this information. The names of participants will not be used for publication in any form. The records will be maintained until the data are published, up to a maximum of five years. All archived samples will be coded, but the key linking the code to each participant's identifiable information will have been destroyed. In addition, participants will be instructed that their participation in this study is voluntary and that they may withdraw at any time without prejudice. Data (biochemical values) obtained from this study will stored on a computer in the PI's laboratory. In addition, a backup of digital data will be stored on the PI's computer in his office. Both computers are password protected and both doors are locked when work areas are not in use.

E. Internal Validity

For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant. This will minimize measurement bias when performing analysis on dietary records, and biochemical markers because all samples/records will be coded. The codes will only be broken once data analysis has been completed and verified by the PI.

F. Medical Safety Plan

All aspects of the clinical study will be conducted in Dr. Bruno's clinical lab located in in 262D Campbell Hall. Participants will be fasted for 10-12 hours prior to each study visit. We recognize that certain risks associated with fasting include: hypoglycemia, weakness, and fainting. This duration of fasting is consistent with guidelines set forth by the American Diabetes Association to minimize risk to the individual[62] when determining fasting blood glucose concentrations.

Risks related to hypoglycemia are anticipated to be low due to many participants' already elevated fasting blood glucose (study entry criteria = 100-126 mg/dL). They will also be ingesting a glucose beverage containing 75 g of glucose immediately following the fasting period. Throughout each visit to the study center, participants will be closely monitored by Sudeep Shrestha, Emily Shaw and Geoffrey Sasaki (Postdoctoral Fellow and Doctoral Students), all of them are members of the research team. They will monitor the safety and well-being of participants for any signs and symptoms of hypoglycemia including: confusion, dizziness, irritability, weakness, headaches, and fainting. Emily

Shaw is certified by the Red Cross in CPR and basic first aid. Additionally, Dr. Bruno (PI; 325 Campbell Hall) has an academic office in close proximity to the study center and has significant experience coordinating clinical research studies involving overnight fasting, and other dietary- and carbohydrate-challenges, thereby supporting the competency of our research team in managing potential adverse events relating to fasting glucose and glycemic responses.

Consistent with our prior studies of similar design, and to ensure participant safety, all procedures throughout each study visit will occur while positioned on a hospital bed in the prone position. In the event that a participant was to become weak, dizzy, or faint, they would already be ideally positioned to minimize risks associated with these symptoms. In the event that hypoglycemia-related symptoms occur, the study would be terminated to allow the participant to recover. We are prepared to provide pre-packaged beverages and snacks containing simple carbohydrates (e.g. Gatorade, fruit juice, apple sauce, crackers) that will allow for rapid restoration of blood glucose. These food items will be stored in a refrigerator, located in close proximity (262 Campbell Hall), that is dedicated for foods used in research studies. Participant status (e.g. attentiveness, skin color) will be monitored in our clinical laboratory to ensure recovery. The clinical area where participants undergo blood draws is also equipped with first-aid measures (e.g. smelling salt) and all study team members are trained to assist with basic first-aid application if needed.

Any adverse hypoglycemic response that might occur during this study will receive care commensurate to the symptoms. For example, if a participant were to faint, then smelling salts would be administered along with a carbohydrate-containing food upon regaining consciousness. The study PI would also be contacted.

Alternatively, should a more severe hypoglycemic response occur (e.g. contusion or laceration relating to fainting), the research team would immediately contact medical services (i.e. 911). For non-life threatening emergencies, any OSU students participating in these studies would be directed to the Wilce Student Health Center. For other emergencies or those participants who are not OSU students, individuals would be directed to the Wexner Medical Center either by transporting them directly or requesting ambulance service. Regardless of the complexity of the adverse events, the research team would monitor the participant in the interim, provide palliative care as appropriate, and follow-up after any medical care has been provided.

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